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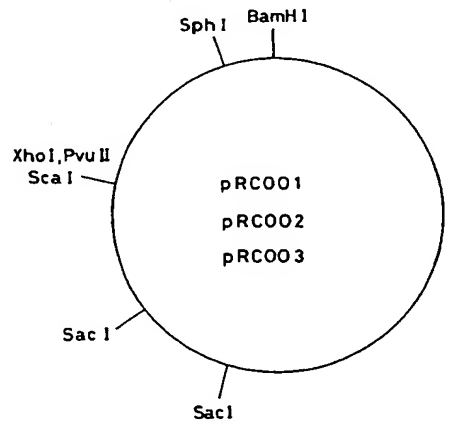
## (54) Novel circular plasmids derived from the genus rhodococcus.

(57) The present invention provides an isolated circular plasmid of about 2.6 kb derived from a bacterium belonging to the genus Rhodococcus whose restriction sites comprises two SacI sites, one BamHI site, one PvuI site, one ScaI site, one SphI site and one XhoI site. In addition, the present invention provides an isolated circular plasmid of about 7.0 kb derived from a bacterium belonging to the genus Rhodococcus whose restriction sites comprises one SphI site, two KpnI sites, one BglII site, and three SacI sites. The vectors of the invention can be suitable for Rhodococcus hosts and useful in industry.

FIG. 1



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The present invention relates to novel plasmids, specifically plasmids derived from bacteria belonging to the genus Rhodococcus.

Bacteria belonging to the genus Rhodococcus have been known to hydrate nitrils to amides or acids. Also, certain strains belonging to Rhodococcus rhodochrous have been known to contain a nitril hydration activity.

However, vectors suitable for Rhodococcus hosts have not been developed yet and, in reality, few vectors are available from very few sources such as Rhodococcus sp. H13-A (J. Bacteriol., 1988, 170: 638 - 645) to date. To utilize the useful properties of the bacteria, vectors suitable for Rhodococcus hosts and subsequent industrial use have been long awaited.

The present inventors have investigated bacteria belonging to Rhodococcus that contain vectors suitable for hosts and industrial use and successfully found circular plasmids suitable as vectors. The present invention provides an isolated circular plasmid of about 2.6 kb derived from a bacterium belonging to the genus Rhodococcus whose restriction sites comprises two SacI sites, one BamHI site, one PvuI site, one ScaI site, one SphI site and one XhoI site. In addition, the present invention provides an isolated circular plasmid of about 7.0 kb derived from a bacterium belonging to Rhodococcus sp. whose restriction sites comprises one SphI site, two KpnI sites, one BglII site, and three SacI sites.

Fig. 1 shows a restriction map of plasmid pRC001, pRC002, and pRC003.

Fig. 2 shows a restriction map of plasmid pRC010.

Novel plasmids, pRC001, pRC002, and pRC003, are derived from Rhodococcus rhodochrous ATCC 4276, ATCC 14349 or ATCC 14348, respectively. The sizes of the plasmids are all about 2.6kb. Table 1 shows numbers of restriction sites and sizes of restriction fragments.

Table 1

| Restriction enzyme | Number of restriction Sites | Size (kb) |
|---|---|---|
| SacI | 2 | 2.3, 0.3 |
| BamHI | 1 | 2.6 |
| PvuII | 1 | 2.6 |
| ScaI | 1 | 2.6 |
| SphI | 1 | 2.6 |
| XhoI | 1 | 2.6 |

A novel circular plasmid pRC010 is derived from Rhodococcus rhodochrous ATCC 4001. The size of the plasmid is about 7.0 kb. Table 2 shows numbers of restriction sites and sizes of restriction fragments.

Table 2

| Restriction enzyme | Number of restriction Sites | Size (kb) |
|---|---|---|
| SphI | 1 | 7.0 |
| KpnI | 2 | 4.0, 3.0 |
| BglII | 2 | 6.5, 0.5 |
| SacI | 3 | 4.2, 2.0, 0.8 |

## Examples

The following example will further describe the invention.

(1) Isolation and Purification of Plasmid

Each of Rhodococcus rhodochrous ATCC 4276, ATCC 14349 or ATCC 14348 was grown in 400 ml of a MY medium (0.5% polypepton, 0.3% bactoyeast extract, 0.3% malt extract, 1% glucose). When $OD_{660}$ reached 0.15 - 0.2, 0.5U/ml of penicillin G was added to the culture. The culture was further incubated until

$OD_{660}$ of 1.0. After incubation, bacterial cells were harvested by centrifugation, washed with 40 ml of a TES buffer(10 mM Tris-HCl/pH 8, 10mM NaCl, 1mM EDTA), and then suspended in 11ml of a solution containing 50mM Tris-HCl/pH 8, 12.5% sucrose, 100mM NaCl, 1mg/ml of lysozyme. The suspension was incubated with shaking at 37°C for 3 hours. 0.6 ml of 0.5M EDTA, 2.4 ml of 5M NaCl, and 4.4 ml of 4% SDS/0.7M NaCl were added in the listed order to the bacterial cell lysate. The mixture was gently swirled and placed on ice for 18 hours. After incubation, the mixture was centrifuged at 4°C, at 65,000 X g for an hour. After centrifugation, the supernatant was saved and then 4.6 ml of 50 % polyethyleneglycol 6,000 was added to the supernatant. The mixture was placed on ice for 3 hours. After incubation, the mixture was centrifuged at 1,000 Xg for 5 minutes. The supernatant was discarded and the pellet was dissolved in 5 ml of a TES buffer and then 2 ml of a TES buffer containing 7.5 g of cesium chloride and 1.5 mg/ml of ethidium bromide was added to the pellet solution. The mixture was ultracentrifuged at 130,000 X g for 42 hours. After ultracentrifugation, the fraction containing plasmids was removed under the UV light. The plasmid fraction was extracted with n-butanol to remove ethidium bromide. After extraction, the plasmid fraction was dialyzed against TE (10 mM Tris- HCl/pH 8, 1mM EDTA) and then precipitated with ethanol. The plasmid thus obtained was electrophoresed on a 0.7 % agarose gel. The gel was stained with ethidium bromide and examined under the UV light. The band containing plasmids was found on the gel.

(2) Determination of Molecular Weight of Plasmid

Plasmids were electrophoresed along with pUC18 (2.69 kb), pUC 118 (3.16 kb), and pBR322 (4.36 kb) as markers on a 0.7 % agarose gel. The sizes of plasmids were all about 2.6 kb. Plasmids were designated as pRC001(ATCC 4276), pRC002 (ATCC 14349) and pRC 003 (ATCC 14348). The parenthesis indicates the ATCC number of the Rhodococcus rhodochrous source.

(3) Numbers and Sizes of Restriction Sites of Plasmids

Plasmids, pRC001, pRC002 and pRC 003, were digested with various restriction enzymes and restriction fragments were electrophoresed along with markers such as HindIII- and PstI-digested lambda phage DNA on 0.7 % agarose gel and 5 % acrylamide gel. The results are shown in Table 3.

Table 3

| Restriction enzyme | Number of restriction Sites | Size (kb) |
|---|---|---|
| SacI | 2 | 2.3, 0.3 |
| BamHI | 1 | 2.6 |
| PvuII | 1 | 2.6 |
| ScaI | 1 | 2.6 |
| SphI | 1 | 2.6 |
| XhoI | 1 | 2.6 |
| EcoRI | 0 | - |
| HindIII | 0 | - |
| KpnI | 0 | - |

Example 2

(1) Isolation and Purification of Plasmid

Rhodococcus rhodochrous ATCC 4001 was grown in 400 ml of a MY medium (0.5% polypepton, 0.3% bactoyeast extract, 0.3% malt extract, 1% glucose). When $OD_{660}$ reached 0.15 - 0.2, 0.5U/ml of penicillin G was added to the culture. The culture was further incubated until $OD_{660}$ of 1.0. After incubation, bacterial cells were harvested by centrifugation, washed with 40 ml of a TES buffer(10 mM Tris-HCl/pH 8, 10 mM NaCl, 1mM EDTA), and then suspended in 11ml of a solution containing 50 mM Tris-HCl/pH 8, 12.5% sucrose, 100 mM NaCl, 1mg/ml of lysozyme. The suspension was incubated with shaking at 37°C for 3 hours. 0.6 ml of 0.5M EDTA, 2.4 ml of 5M NaCl, and 4.4 ml of 4% SDS/0.7M NaCl were added in the listed order to the bacterial cell lysate. The mixture was gently swirled and placed on ice for 18 hours. After

incubation, the mixture was centrifuged at 4°C, at 65,000 X g for an hour. After centrifugation, the supernatant was saved and then 4.6 ml of 50 % polyethyleneglycol 6,000 was added to the supernatant. The mixture was placed on ice for 3 hours. After incubation, the mixture was centrifuged at 1,000 Xg for 5 minutes. The supernatant was discarded and the pellet was dissolved in 5 ml of a TES buffer and then 2 ml of a TES buffer containing 7.5 g of cesium chloride and 1.5 mg/ml of ethidium bromide was added to the pellet solution. The mixture was ultracentrifuged at 130,000 X g for 42 hours. After ultracentrifugation, the fraction containing plasmids was removed under the UV light. The plasmid fraction was extracted with n-butanol to remove ethidium bromide. After extraction, the plasmid fraction was dialyzed against TE (10 mM Tris- HCl/pH 8, 1mM EDTA) and then precipitated with ethanol. The plasmid thus obtained was electrophoresed on a 0.7 % agarose gel. The gel was stained with ethidium bromide and examined under the UV light. The band containing plasmids was found on the gel.

(2) Determination of Molecular Weight of Plasmid

Plasmids were electrophoresed along with pUC18 (2.69 kb), pUC 118 (3.16 kb), and pBR322 (4.36 kb) as markers on a 0.7 % agarose gel. The size of plasmids was about 7.0 kb. The plasmid was designated as pRC010 (ATCC 4001). The parenthesis indicates the ATCC number of the Rhodococcus rhodochrous source.

(3) Numbers and Sizes of Restriction Sites of Plasmids

Plasmid pRC010 was digested with various restriction enzymes and restriction fragments were electrophoresed along with markers such as HindIII- and PstI-digested lambda phage DNA on 0.7 % agarose gel and 5 % acrylamide gel. The results are shown in Table 4.

Table 4

| Restriction enzyme | Number of restriction Sites | Size (kb) |
|---|---|---|
| SphI | 1 | 7.0 |
| KpnI | 2 | 4.0, 3.0 |
| BglII | 2 | 6.5, 0.5 |
| SacI | 3 | 4.2, 2.0, 0.8 |
| BamHI | 0 | - |
| BclI | 0 | - |
| EcoRI | 0 | - |
| HindIII | 0 | - |
| ClaI | 0 | - |
| PvuII | 0 | - |
| PstI | 0 | - |
| ScaI | 0 | - |
| SmaI | 0 | - |

## Claims

1. An isolated circular plasmid of about 2.6 kb derived from a bacterium belonging to the genus Rhodococcus whose restriction sites comprises two SacI sites, one BamHI site, one PvuI site, one ScaI site, one SphI site and one XhoI site.

2. The isolated circular plasmid of claim 1 comprising any one of plasmids derived from Rhodococcus rhodochrous ATCC 4276, ATCC 14349, and ATCC 14348.

3. An isolated circular plasmid of about 7.0 kb derived from a bacterium belonging to the genus Rhodococcus whose restriction sites comprises one SphI site, two KpnI sites, one BglII site, and three SacI sites.

4. The isolated circular plasmid of claim 2 comprising any one of plasmids derived from Rhodococcus rhodochrous ATCC 4001.

FIG. 1

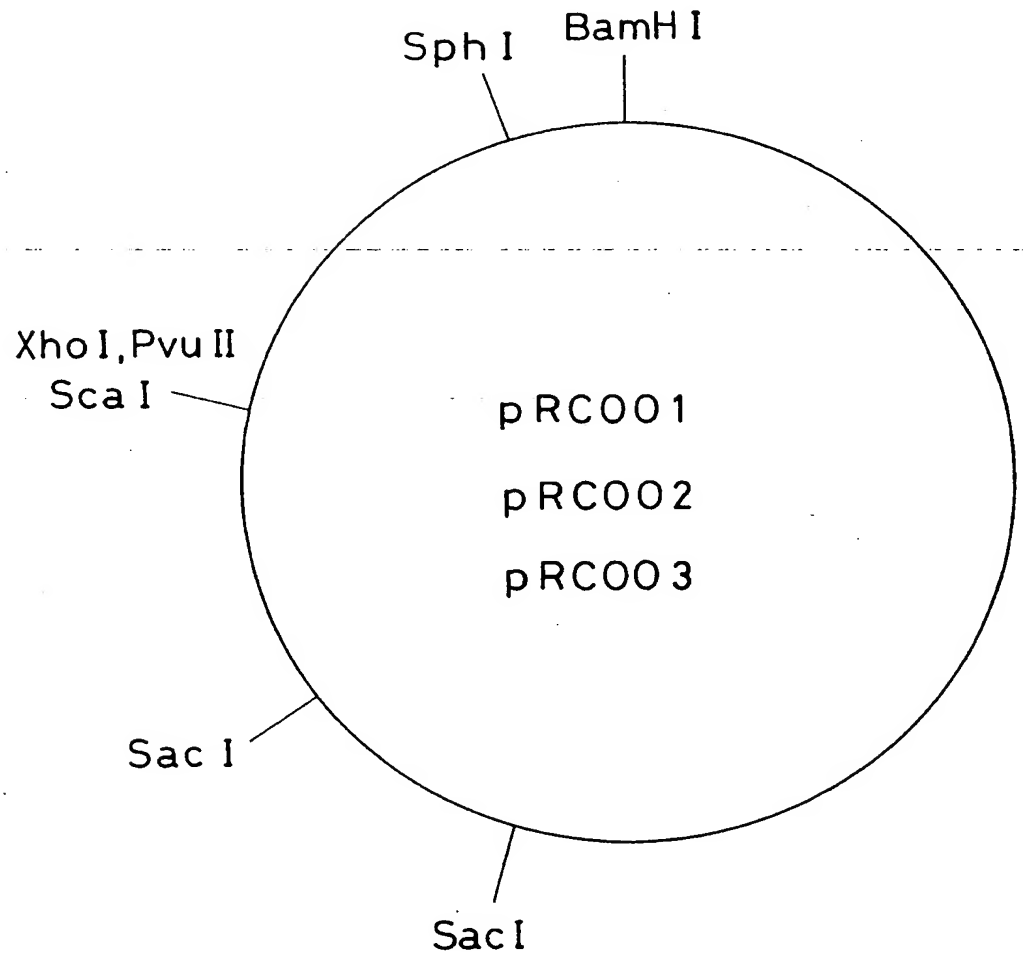
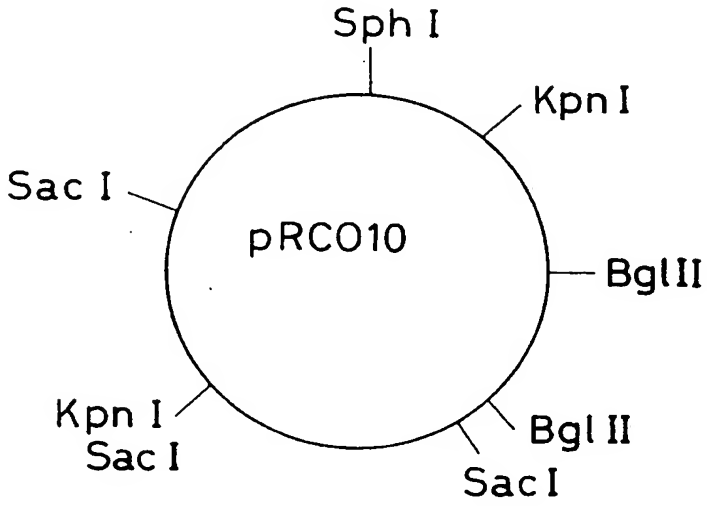


FIG. 2



European Patent Office

# EUROPEAN SEARCH REPORT

Application Number

EP 91 11 7212

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.5) |
|---|---|---|---|
| A | WO-A-8 907 151 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC.)<br>* abstract * | 1-4 | C12N15/74<br>C12N15/10 |
| A | US-A-4 920 054 (KOZLOWSKI M. ET AL)<br>* page 3, line 3 - line 4 *<br>* page 3, line 46 - page 4, line 7 * | 1-4 | |
| A | BIOLOGICAL ABSTRACTS, volume 85, no. 1, 1988<br>KUSAKA, TAIKI et al: "Plasmid-possessing actinomycetes in IFO", abstract no. 3791<br>& INST. FERMENT. RES. COMMUN.<br>vol. 0, no. 13, 1987,<br>pages 5 - 12; | 1-4 | |
| A | BIOLOGICAL ABSTRACTS, volume 89, no. 7, 1990,<br>MIL'KO, E.S. et al: "The genetic basis of dissociative transition in Rhodococcus rubropertinctus." abstract no. 70218.<br>& VESTN. MOSK. UNIV. SER. XVO BIOL.<br>vol. 0, no. 3, 1989,<br>pages 40 - 44; | 1-4 | TECHNICAL FIELDS SEARCHED (Int. Cl.5) |
| A | BIOLOGICAL ABTRACTS, volume 85, no. 8, 1988,<br>DABBS, E. R. et al: "Plasmid-borne resistance to arsenate, arsenite, cadmium, and chloramphenicol in a Rhodococcus sp." abstract no. 78582<br>& MOL. GEN. GENET.<br>vol. 211, no. 1, 1988,<br>pages 148 - 154; | 1-4 | C12N |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 03 MARCH 1992 | LE CORNEC N.D.R. |

CATEGORY OF CITED DOCUMENTS

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